

The Masculinization of the Fetus During Pregnancy Due to Inhalation of Diesel Exhaust

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This study was conducted to determine the impact of diesel exhaust inhalation on the fetus. Seventy-two pregnant rats and 18 nonpregnant rats were divided into three groups: a group exposed to total diesel engine exhaust containing 5.63 mg/m³ particulate matter, 4.10 ppm nitrogen dioxide, and 8.10 ppm nitrogen oxide; a group exposed to filtered exhaust without particulate matter; and a group exposed to clean air. The exposure period was from day 7 until day 20 of pregnancy. In addition, 15 pregnant rats were treated with aromatase inhibitors or testosterone to clarify the process by which diesel exhaust exerts its toxicity. The anogenital distance was significantly longer in male and female fetuses from both exhaust-exposed groups than in those of the control. Differentiation of the testis, ovary, and thymus was delayed and disturbed. Maternal testosterone and progesterone levels, which increased due to pregnancy whether or not the rats were exposed, were significantly higher and lower, respectively, in the pregnant rats exposed to total exhaust and filtered exhaust. The serum adrenocorticotropic hormone (ACTH) level and urinary excretion of 17-hydroxycorticosteroids (OHCS) did not differ among the pregnant groups. These results indicate that elevated testosterone did not result from elevated maternal adrenal function. The fetoplacental-ovarian unit and inhibition of aromatase activity and synthesis caused by diesel exhaust inhalation might have played an essential role in the accumulation of testosterone. Since both exhaust-exposed groups showed almost the same reactions toward the inhalation, the gaseous phase must have included the relevant toxicants. **Key words:** diesel exhaust, fetoplacental-ovarian unit, fetus, masculinization, ovary, pregnancy, rats, testis, testosterone, thymus. *Environ Health Perspect* 109:111–119 (2001). [Online 11 January 2001] <http://ehpnet1.niehs.nih.gov/docs/2001/109p111-119watanabe/abstract.html>

In growing rats, inhalation of diesel engine exhaust has been shown to depress the secretion of gonadotropin-releasing hormone and inhibit spermatogenesis through the stimulation of steroid hormone secretion by the adrenal cortex, both directly and indirectly (1,2). Multiple studies suggest that inappropriate exposure to sex hormones and chemicals during critical stages of sex differentiation can disrupt the normal sequence of maturation and cause reproductive dysfunction (3–5). Although emissions from diesel exhaust contain a variety of chemicals and oxidative gases, adverse effects of inhalation of diesel exhaust have not been proven to occur during growth and development.

It is well known that fetuses and neonates are exposed to exogenous endocrine disrupters through the placenta and mother's milk (6–11). In addition to these direct transfers of toxic substances from mothers, endogenous sex steroids accumulated to excess in mothers due to enzymatic deficiency can also disrupt differentiation of reproductive organs in offspring. Aromatase deficiency caused by mutations in the *CYP19* gene leads to excess circulation of androgens and results in virilization and ambisexual development (12–15). Prenatal exposure to nicotine (16–18) and several other drugs (19) reduces or suppresses aromatase activities as well. Nitric oxide also produces inhibitory effects on the expression and activity of aromatase in human granulosa

cells (20,21). Transport of endogenous sex steroids produced in the process of physiological differentiation from one fetus to another might be one of the other factors that disrupts fetal differentiation and neonate development (22–25). Excessive steroid hormones, of both exogenous and endogenous origin, are toxic in the early steps of T-cell differentiation (26–29). T-cell deficiency elevates serum IgE in infants (30), which has a strong connection with allergic diseases (31). These findings strongly suggest that inhalation of diesel exhaust during pregnancy could disrupt the development of reproductive organs and the immune system through the accumulation of endogenous steroid hormones.

We conducted this study to pursue three objectives. First, we sought to determine the impact of diesel exhaust inhalation on the fetus during pregnancy. Exposure began on day 7 of pregnancy to prevent the effects of inhalation on the fertilized egg before implantation (32). On day 20, we measured several parameters, such as litter size, implantation rate, and the number of males and females as determined by visual appearance and anogenital distances, which reflect the levels of masculinization. In addition, the fetal reproductive organs and thymus were examined histologically. Our second objective was to determine whether increased or decreased maternal serum hormone levels are related to morphologic changes of the fetus.

Our third objective was to determine the mechanism by which diesel inhalation exerts its toxicity by administering aromatase inhibitors or testosterone to pregnant rats.

Materials and Methods

Experiment 1

Seventy-two pregnant Fischer rats (F344/DuCrj) obtained from Charles River Japan (Kanagawa, Japan), 24 rats per group, were divided into three groups. Group 1 was exposed to total diesel engine exhaust (total exhaust), group 2 was exposed to filtered exhaust without particles (filtered exhaust), and group 3 was exposed to clean air (control). Each group of animals was maintained in an inhalation chamber (1.6 m³) at 24 ± 2°C and 55 ± 5% humidity on a 12 hr light:12 hr dark illumination schedule. The diet was standard rat chow containing 1.03% calcium, 0.70% phosphorus, and 200 IU vitamin D₃/100 g (MF, Oriental Yeast Co. Ltd, Tokyo, Japan). All animals were allowed free access to food and water. Exposures began on day 7 (day of impregnation = day 0) and continued until day 20 of pregnancy. The exposure period was 6 hr daily.

At the end of the experiment, we measured body weights and collected blood samples from the abdominal aorta with the rats under ether anesthesia. After the mother rats were killed by exsanguination, the two uterine horns were spread out and pups were removed from the horns and laid out according to their uterine position. After recording the position, we removed the fetuses and measured their body weights and the weight of the placenta. We measured the distance from the center of the anus to the genital orifice (anogenital distance) using a dissecting microscope with a micrometer disc according to the methods of vom Saal et al. (23). We determined the sex phenotype according to the visual appearance of the reproductive organs. We examined the numbers of implantation traces in the uteri—including traces causing early embryonal death, abortion, and normal embryos—to assess the

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Received 7 July 2000; accepted 22 August 2000.

survival rate, by staining with 10% sodium hydroxide using the method described by Yamada et al. (33,34).

To determine the effects of the intrauterine position of fetuses on anogenital distance, we classified female fetuses as 2M, 1M, and 0M females (2M = between 2 males, 1M = between a male and a female, and 0M = between 2 females) and males as 2F, 1F, and 0F males (2F = between 2 females, 1F = between a male and a female, and 0F = between 2 males), according to the method of vom Saal et al. (23).

Generation of diesel exhaust. Diesel engine exhaust was generated by running a 309 cc engine (Model NFAD50; Yanmar Diesel Co., Osaka, Japan) at 2,400 rpm. Exhaust was diluted with clean air in a dilution tunnel and then drawn into the inhalation chamber (particulate matter = 5.63 mg/m³, nitrogen dioxide = 4.10 ppm, nitrogen oxide = 8.10 ppm). For the filtered group, most of the diesel soot particles in whole exhaust were removed by HEPA filtration (ATM 3QA; Nippon Muki Co., Tokyo, Japan). After filtration, 99.9998% of particles measuring 0.05 µm or more were eliminated. Ventilation was maintained by 15 air exchanges/hr. Concentrations of nitrogen dioxide and nitrogen monoxide were monitored continuously with a chemiluminescent analyzer (Model 8440; Monitor Labs Co., San Diego, CA, USA). Gravimetric measurements of the particulate matter were conducted daily using an automatic beta-ray dust-mass monitor (Model BAM-102; Shibata Scientific Technology Co., Tokyo, Japan). Measurement of particle sizes with a particle fractionating sampler (Andersen Type low pressure impactor LP-20; Tokyo Dylec Co., Tokyo, Japan) confirmed that more than 90% of the particulate matter in the diesel exhaust measured less than 0.5 µm.

Histologic preparation. On day 20 of pregnancy, gonads and thymus of fetuses were removed and fixed in formalin. The tissue was imbedded in paraffin, cut into 4-µm sections, stained with hematoxylin-eosin, and examined.

Serum hormonal assay. Serum testosterone, progesterone, and estradiol levels of mother rats were determined using Enzyme Immunoassay Kits (Cayman Chemical, Ann Arbor, MI, USA). We determined serum levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) using a rat LH enzyme immunoassay system and a rat FSH enzyme immunoassay system (Amersham, Buckinghamshire, England), respectively. Serum levels of adrenocorticotropic hormone (ACTH) were determined using a rat ACTH high-sensitivity enzyme immunoassay kit (Peninsula Laboratories, San Carlos, CA, USA).

Urine measurement of excretion of 17-ketosteroids (17-KS) and 17-hydroxycorticosteroids (17-OHCS). Experimental animals were placed into individual metabolic cages to collect overnight urine samples (1600–2100 hr) on the 16th day of pregnancy.

Urinary 17-KS and 17-OHCS concentrations, markers of adrenal cortex function, were measured using the diagnostic kits OS-KIT and Oha-KIT (Kanto Chemicals, Tokyo, Japan). We measured creatinine (CRE) levels in urine using a diagnostic kit (Daiichi Pure Chemicals, Tokyo, Japan) and an automatic analyzer (Model Hitachi 705; Hitachi, Tokyo, Japan). The results were expressed as 17-KS/CRE and 17-OHCS/CRE ratios (micrograms per milliliter/CRE) in spontaneous urine samples.

Experiment 2

To determine the serum hormone changes resulting from pregnancy, we measured serum hormones of nonpregnant and postpartal rats. Eighteen nonpregnant female rats corresponding with the pregnant rats used in experiment 1 were divided into three groups; total exposed, filtered exposed, and control. After a 14-day exposure to each type of air, we assayed serum hormones using the same methods described above for experiment 1.

Eighteen pregnant rats were divided into three groups: total exposed, filtered exposed, and control. The exposure period was from day 7 of pregnancy until 3 weeks after delivery. At the end of the experiment, we collected and assayed blood samples.

Experiment 3

Fifteen pregnant rats were housed and kept on a 12 hr light:12 hr dark schedule. The clean room was maintained at 24 ± 2°C and 55 ± 5% humidity. On day 20 of pregnancy after administration of either aromatase inhibitor or testosterone, we collected blood samples from the abdominal aorta and serum hormones; we removed the pups from the uterine horns, measured body weight, placental weight, and anogenital distance, and determined the sex phenotype using the same methods described above.

Administration of aromatase inhibitor. From day 7 until day 20 of pregnancy, six pregnant rats were injected daily subcutaneously with fadrozole, -4-(5,6,7,8-tetrahydroimidazo[1,5-α]-pyridin-5-yl) benzonitrile monohydrochloride (Novartis-Pharma Co., Tokyo, Japan), at a dose of 0.25 mg/kg in 0.1 mL of 0.9% NaCl solution (aromatase inhibitor group).

Administration of testosterone. **Testosterone group 1.** From day 7 until day 20 of pregnancy, six pregnant rats were injected daily sc with testosterone propionate

(Wako, Chemicals, Tokyo, Japan) at a dose of 1.5 mg/kg in 0.1 mL of sesame oil.

Testosterone group 2. Three pregnant rats were injected daily with testosterone propionate from day 12 until day 20 of pregnancy with the same dose as testosterone group 1. We began administration on day 12 of pregnancy to avoid fetal death, which had occurred in testosterone group 1. The placenta begins secreting testosterone on day 10 of pregnancy (35), and the testes in male embryos differentiate and begin secreting testosterone around the second week of gestation in rats (36–38). Our timing was designed to ensure that the administered testosterone could act before the hypothalamic-pituitary-gonad axis of the fetuses began to function, which is thought to occur around day 15 of pregnancy (35,39).

Statistical analysis. All reported values are expressed as means ± standard deviations (SD). We analyzed the differences between the three groups using one-way analysis of variance (ANOVA) and the Student's *t*-test with a Bonferroni correction. We used SPSS statistical software (SPSS Inc., Chicago, IL, USA) for the analyses. We considered *p*-values < 0.05 significant.

The treatment and care of the rats was carried out according to a protocol approved by the Animal Care and Use Committee of the Tokyo Metropolitan Research Laboratory of Public Health in a facility approved by the Japan Association for Accreditation of Laboratory Animal Care.

Results

Fetal Parameters

Each group contained 24 pregnant rats at the beginning of the experiment. Ten of these pregnant rats—four each in the control group and the group exposed to filtered exhaust and two in the the group exposed to total exhaust, which had been verified as pregnant by the presence of a vaginal plug—appeared to be nonpregnant at the end of the experiment. There were no implantation traces in their uteri. We excluded these nonpregnant rats from data analysis. The average body weight of pregnant rats did not differ among the groups. Litter sizes ranged from 6 to 11, and the average litter sizes in the total-exhaust group, filtered-exhaust group, and control group were 8.36, 8.80, and 8.75, respectively. The number of fetuses and the sex ratio of litters in the total-exhaust group, filtered-exhaust group, and control group were 183 (male:female = 98:85, males = 53.6%), 174 (77:97, 44.3%), and 175 (95:80, 54.3%), respectively. The implantation rate in the total-exhaust group, filtered-exhaust group, and control group was 94.8, 96.2, and 95.6, respectively. There were no

significant differences in litter size, sex ratio, and implantation rate among these groups.

The average body weight, average weight of the placenta, anogenital distance, and average weight of the thymus in fetuses from rats exposed to diesel exhaust and clean air are shown in Table 1. The average body weight was greater in exhaust-exposed female fetuses than in those of the control group ($p < 0.001$). The average weight of the placenta was lower in total-exhaust-exposed male fetuses than in those of the filtered group and control group ($p < 0.05$). The anogenital distance was significantly longer in the total-exhaust-exposed and filtered groups than in the control group in both sexes ($p < 0.001$). There was no difference in anogenital distance between those exposed to total exhaust and to filtered exhaust. Body weight variation did not contribute significantly to the variance in anogenital distance. The average weight of the thymus was significantly lower in both exhaust-exposed groups than in the control group both in males and females ($p < 0.001$). There were no differences in thymus weight between the groups exposed to total exhaust and to filtered exhaust. There were no differences in implantation rate among groups. There was no evidence of congenital malformation in fetuses from mothers exposed to diesel exhaust.

Morphologic Changes

Microscopic examination revealed that differentiation of the fetal testis was delayed or disturbed markedly in the fetuses from the groups exposed to total and filtered exhaust. In the control group, the fetal testis contained cells of two types. The larger were primordial germ cells that act as stem cells, proliferate, and become spermatogonia. The smaller were Sertoli cells, located in the periphery of the seminiferous tubules. There were interstitial cells, which are responsible for the endocrine secretion, in the unspecialized connective tissue between the seminiferous tubules (Figure 1). There were fewer Sertoli cells in the testes of fetuses from exposed mother rats. Primordial germ cells and undifferentiated cells, which transform ultimately into sustentacular cells of Sertoli, were seen in the seminiferous tubules (Figure 2).

Histologic examination showed that the differentiation of the fetal ovaries from exhaust-exposed rats was delayed compared to that in fetuses from control-group rats. The number of germ cells growing in the primary medulla and cortex decreases with the addition of new cells (secondary cortex) at the periphery of the ovary. Young primary follicles were occasionally observed in the secondary cortex in the ovaries from control rats (Figure 3). In the exhaust-exposed

groups, there were still numerous germ cells growing in the primary medulla and cortex in the fetal ovaries (Figure 4).

Histologic examination showed that the differentiation of the fetal thymus from the exhaust-exposed rats was delayed compared to that of the thymus from fetuses from control-group rats. The control group showed numerous lymphocytes in the thymus with uniform appearance. Thymic epithelial cells were hard to detect. Mature thymocytes did not appear on fetal day 20 (Figure 5). There were fewer lymphocytes in the thymuses of fetuses in the exposed groups than in those of fetuses in the control group. These lymphocytes were distributed sparsely, and thymic epithelial cells were observed (Figure 6).

Influence of Intrauterine Position on Anogenital Distance

Figure 7 shows the relationship between the anogenital distance in females and the fetal position in the uterus. In the control group, the anogenital distance tended to be longer in 2M females than in 0M and 1M females, although the influence of intrauterine position on anogenital distance was not significant. In the exhaust-exposed groups, the anogenital distance was influenced by the inhalation, but intrauterine position did not seem to affect the distance. Figure 8 shows

Table 1. Parameters of fetuses from mother rats exposed to diesel exhaust or clean air and from mother rats administered either aromatase inhibitor or testosterone.

	Average body weight of fetuses (g)		Average weight of placenta (g)		Anogenital distance (mm)		Average weight of thymus (mg)	
	Males	Females	Males	Females	Males	Females	Males	Females
Exposed group								
Clean air	3.42 ± 0.23	3.13 ± 0.20	0.51 ± 0.06	0.52 ± 0.07	3.58 ± 0.33	2.59 ± 0.26	7.3 ± 1.1	7.6 ± 0.8
Filtered exhaust	3.45 ± 0.25	3.27 ± 0.22**	0.51 ± 0.06	0.52 ± 0.07	4.10 ± 0.35**	3.01 ± 0.33**	6.0 ± 0.7**	6.1 ± 0.7**
Total exhaust	3.40 ± 0.24	3.24 ± 0.18**	0.49 ± 0.05*#	0.53 ± 0.08	3.98 ± 0.45**	3.01 ± 0.41**	6.2 ± 0.7**	6.2 ± 1.0**
Administered group								
Aromatase inhibitor	3.51 ± 0.24	3.32 ± 0.18	0.71 ± 0.10	0.72 ± 0.08	4.41 ± 0.30	3.27 ± 0.30	6.0 ± 0.6	5.3 ± 0.9
Testosterone group 1	2.96 ± 0.34	2.85 ± 0.36	0.47 ± 0.06	0.49 ± 0.05	4.18 ± 0.30	3.30 ± 0.30	4.7 ± 0.5	4.4 ± 1.0
Testosterone group 2	3.33 ± 0.24	3.16 ± 0.27	0.44 ± 0.06	0.49 ± 0.05	4.30 ± 0.23	3.49 ± 0.24	5.8 ± 0.7	6.1 ± 1.0

Values are expressed as mean ± SD. p -Values were corrected by Bonferroni's inequality.

*Different from clean air; $p < 0.05$; **different from clean air, $p < 0.001$; #different from filtered exhaust, $p < 0.05$.

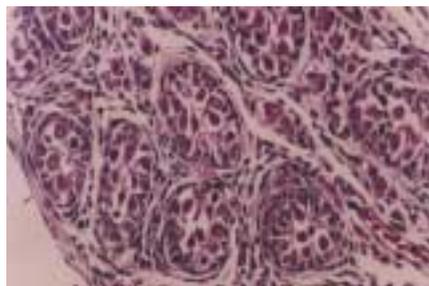


Figure 1. Fetal testis from control group on fetal day 20. The testis contained two types of cells. The larger were primordial germ cells, which act as stem cells, proliferate, and become spermatogonia. The smaller were Sertoli cells, and they were located in the periphery of the seminiferous tubules. There were interstitial cells in the unspecialized connective tissue between the seminiferous tubules. Magnification x100.

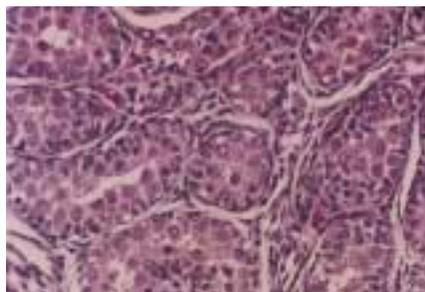


Figure 2. Fetal testis from total-exhaust-exposed group. There were fewer differentiated Sertoli cells. Primordial germ cells and undifferentiated cells were seen in the seminiferous tubules. Differentiation of the fetal testis was delayed or markedly disturbed compared with the testes from the control group. Magnification x100.

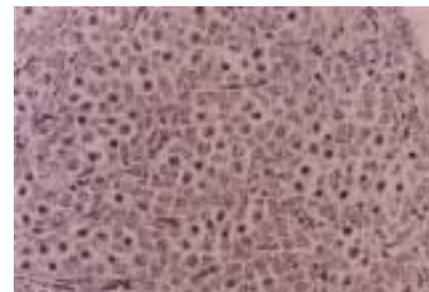


Figure 3. Fetal ovary from control group. The number of germ cells that were growing in the primary medulla and cortex decreased with the addition of new cells (secondary cortex) at the periphery of the ovary. Young primary follicles were occasionally observed in the secondary cortex. Magnification x100.

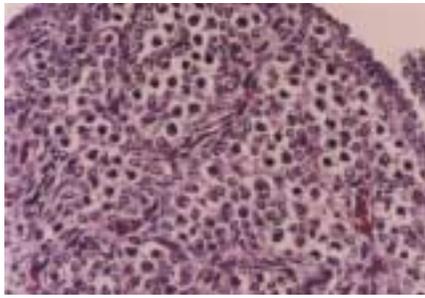


Figure 4. Fetal ovary from total-exhaust-exposed group. There were still numerous germ cells growing in the primary medulla and cortex in the fetal ovary. The differentiation stage of fetal ovaries from diesel-exhaust-exposed rats was delayed compared to that of fetal ovaries from the control group. Magnification x100.



Figure 5. Fetal thymus of the control group. There were numerous cells in the thymus. Proliferating cells were not seen, and mature thymocytes were not detected on fetal day 20. Magnification x100.



Figure 6. Fetal thymus of the exhaust-exposed group. There were fewer cells in the thymus compared to the control. Epithelial cells remained in place. Magnification x100.

the relationship between the anogenital distance in males and the fetal position in the uterus. Intrauterine position did not affect the anogenital distance in the control group. 0F males tended to have a longer anogenital distance than 1F and 2F males in the exposed groups, although there were no significant differences.

Serum Hormone Levels of Pregnant, Nonpregnant, and Postpartum Rats

Table 2 shows serum hormone levels from pregnant rats exposed to diesel exhaust and from control pregnant rats. The table also shows the serum hormone levels of nonpregnant rats of the same ages as the pregnant rats used in experiment 1, the postpartum rats 3 weeks after delivery, and the pregnant rats given aromatase inhibitor or testosterone. The serum levels of testosterone, estradiol, progesterone, and ACTH were markedly elevated with pregnancy in all three groups. The testosterone levels of the exhaust-exposed rats were significantly higher than those of control rats ($p < 0.001$ and $p < 0.01$ for total and filtered exhaust, respectively). We found no correlation between maternal testosterone level and the number of males in the litter or litter size. The estradiol levels of the rats exposed to filtered exhaust were significantly lower than those of the control rats ($p < 0.01$). Although the differences among pregnant rat groups were not significant, the progesterone levels of the exhaust-exposed rats were lower than those of control rats. Serum levels of LH increased slightly with pregnancy regardless of exposure to diesel exhaust. The LH levels of the pregnant rats exposed to filtered exhaust were significantly lower than those of control rats ($p < 0.001$). Serum levels of FSH decreased with pregnancy regardless of exposure to diesel exhaust. The nonpregnant rats exposed to diesel exhaust or clean air for 14 days showed no significant differences among the three groups.

Serum hormone levels of testosterone were higher in postpartum rats exposed to filtered exhaust than in the control rats ($p <$

0.001). There was also a significant difference in testosterone level between the total-exhaust group and the filtered-exhaust group ($p < 0.01$). The estradiol levels of the filtered-exhaust group was significantly lower than those of the control group ($p < 0.05$).

Adrenal and Ovarian Functions of Pregnant and Nonpregnant Rats

Table 3 shows the levels of maternal urinary excretion of 17-KS, which is a marker of adrenal cortex and gonad functions. Urinary excretion of 17-KS increased markedly as a result of pregnancy. 17-KS in the total-exhaust-exposed group tended to be higher than in the other groups, although the differences were not significant. Urinary excretion of 17-OHCS, another marker of adrenal cortex function, increased markedly as a result of pregnancy and did not differ among the pregnant groups.

The thymus weights, average weight of the adrenal glands, and average weight of the ovaries are shown in Table 3. Among pregnant rats, the average weight of the adrenal glands was lower in those exposed to filtered exhaust than in those exposed to total exhaust and in the control pregnant group ($p < 0.05$ and $p < 0.01$ for total exhaust and control, respectively). The thymus weights in exhaust-exposed pregnant rat groups were lower than the weight in the control pregnant rat group ($p < 0.001$ for each). The weight of ovaries in exhaust-exposed pregnant rat groups was lower than the weight in the control pregnant rat group ($p < 0.01$ for each).

Histologic examination of the ovaries on day 20 of pregnancy showed the functional differences of ovary. In the control group, the large, pregnant *corpora lutea* occupied most of the ovary, and around these *corpora lutea* were many primary and secondary follicles as well as the interstitial glands (Figure 9). The pregnant *corpora lutea* in the exhaust-exposed rats were smaller than in those of the control rats. Some of these pregnant *corpora lutea* showed regressive change (Figure 10).

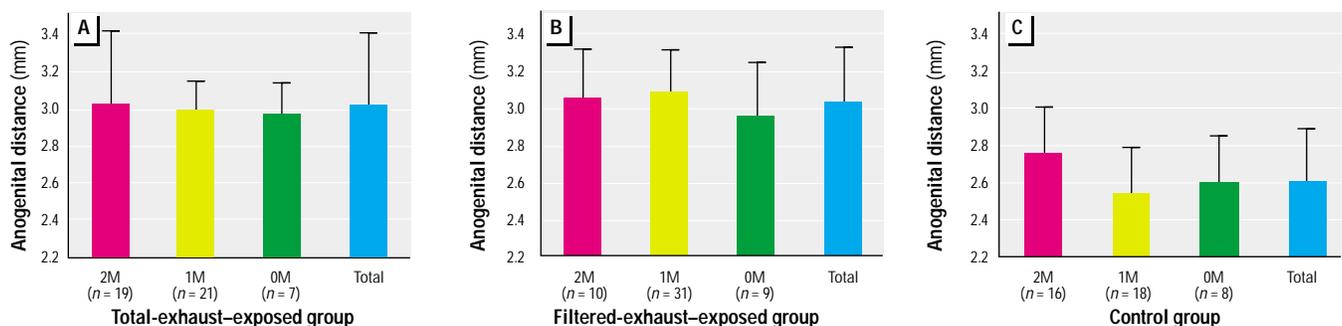


Figure 7. Mean anogenital distance of female fetuses from the diesel-exhaust-exposed rats and the control rats on day 20 of gestation that developed between two male fetuses (2M), next to one male fetus (1M), or next to no male fetuses (0M). "Total" refers to the combined mean for all 0M, 1M, and 2M females measured.

Effects of Aromatase Inhibitor or Testosterone Administration

Six pregnant rats comprised the group given aromatase inhibitor at the beginning of the experiment. One of these pregnant rats, which had been verified as pregnant by the presence of a vaginal plug, appeared to be nonpregnant at the end of the experiment. There were no implantation traces in the uteri. Data shown in Tables 1 and 2 were compiled excluding this nonpregnant rat.

Litter sizes ranged from 7 to 10, and the average litter size in the group given aromatase inhibitor was 8.80. There were 44 fetuses in the aromatase inhibitor group, and the sex ratio of litters was male:female = 20:24 (males = 45.5%). The implantation rate was 93.8.

The fetal parameters in the aromatase-inhibitor group, such as average body weight, anogenital distance, and average weight of the thymus, showed the same tendencies as those in the diesel-exhaust-exposed pregnant

rat groups (Table 1). Placental weight tended to be greater than in the other pregnant groups. Histologic changes of the fetal testes were more severe than those of the testes from the diesel-exhaust-exposed groups and the control group. There were few differentiated Sertoli cells. Swollen primordial germ cells and undifferentiated cells were seen in the seminiferous tubules (Figure 11). The differentiation stage of fetal ovaries from the aromatase inhibitor group was delayed

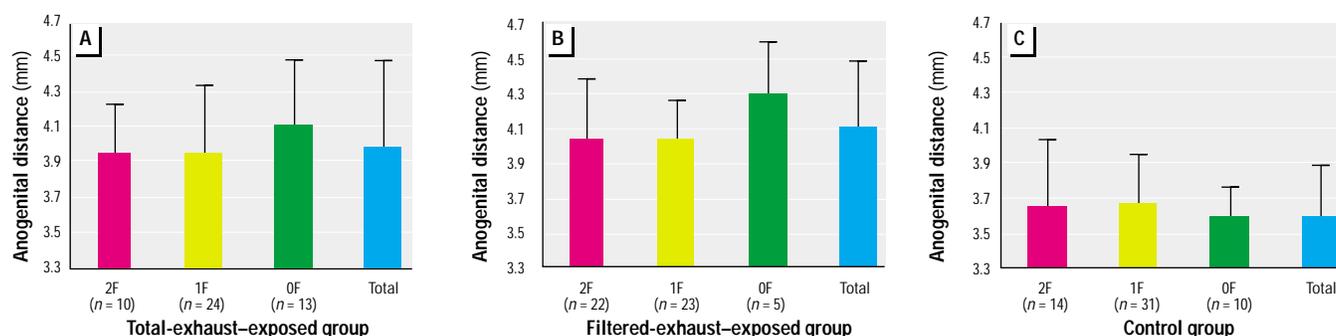


Figure 8. Mean anogenital distance of male fetuses from the diesel-exhaust-exposed rats and the control rats on day 20 of gestation that developed between two female fetuses (2F), next to one female fetus (1F), or next to no female fetuses (0F). "Total" refers to the combined mean for all 0F, 1F, and 2F males measured.

Table 2. Serum hormone levels of pregnant rats, nonpregnant rats, and postpartum rats exposed to either diesel exhaust or clean air, and administered pregnant rats.

Group	No. of rats	Testosterone (pg/mL)	Estradiol (pg/mL)	Progesterone (ng/mL)	FSH (ng/mL)	LH (ng/mL)	ACTH (ng/mL)
Exposed group							
On day 20 of pregnancy							
Clean air	20	228.6 ± 71.4	298.7 ± 93.0	123.9 ± 51.1	193.1 ± 53.6	28.3 ± 4.0	3.0 ± 1.4
Filtered exhaust	20	303.0 ± 70.4***	231.3 ± 43.3***	98.0 ± 49.7	163.5 ± 40.2	22.8 ± 3.9**	2.8 ± 1.1
Total exhaust	22	342.2 ± 98.9**	283.0 ± 99.9	90.7 ± 35.6	171.1 ± 37.8	25.6 ± 3.6	3.1 ± 1.5
Nonpregnant rats exposed for 14 days							
Clean air	6	28.0 ± 6.5	124.4 ± 55.8	3.5 ± 0.8	359.6 ± 77.1	21.1 ± 1.7	1.3 ± 0.2
Filtered exhaust	6	31.4 ± 9.3	109.0 ± 37.8	3.4 ± 0.5	228.2 ± 89.0	17.3 ± 6.2	1.3 ± 0.2
Total exhaust	6	26.5 ± 5.9	127.0 ± 22.8	3.7 ± 1.3	309.1 ± 95.2	21.3 ± 1.5	1.2 ± 0.2
Three weeks after delivery							
Clean air	6	40.9 ± 5.7	160.6 ± 32.5	15.6 ± 11.1	178.5 ± 22.4	15.4 ± 2.1	2.8 ± 0.5
Filtered exhaust	6	74.8 ± 8.6**	122.1 ± 12.1*	11.8 ± 6.2	171.2 ± 49.7	16.7 ± 6.5	3.0 ± 1.0
Total exhaust	6	51.3 ± 12.5##	157.7 ± 52.6	15.2 ± 4.6	218.6 ± 27.3	14.1 ± 1.6	2.4 ± 1.1
Administered pregnant group							
Aromatase inhibitor	5	431.8 ± 65.6	176.0 ± 84.7	80.3 ± 23.7	189.2 ± 64.3	22.6 ± 4.8	1.9 ± 0.3
Testosterone group 1	3	328.8 ± 10.6	77.8 ± 15.0	55.6 ± 34.0	160.4 ± 55.2	17.2 ± 7.0	2.7 ± 1.6
Testosterone group 2	3	326.4 ± 12.3	75.7 ± 23.2	88.6 ± 4.3	165.3 ± 38.3	24.2 ± 2.1	2.8 ± 0.6

Values are expressed as mean ± SD. *p*-Values were corrected by Bonferroni's inequality.

*Different from clean air, *p* < 0.05; ***different from clean air, *p* < 0.01; **different from clean air, *p* < 0.001; ##different from filtered exhaust, *p* < 0.01.

Table 3. Urinary excretion of 17-ketosteroids (17-KS) and 17-hydroxycorticosteroids (17-OHCS), and weight of adrenal glands, thymus, and ovaries from pregnant rats and nonpregnant rats.

Group	No. of rats	17-KS (µg/mL/CRE)	17-OHCS (µg/mL/CRE)	Average weight of adrenal glands (mg)	Average weight of thymus (mg)	Average weight of ovaries (mg)
Pregant group						
Clean air	20	6.8 ± 0.4	8.2 ± 1.1	49.0 ± 2.7	185.1 ± 15.9	114.8 ± 9.1
Filtered exhaust	20	6.7 ± 0.7	8.5 ± 0.5	45.9 ± 3.2***	120.7 ± 13.7**	105.4 ± 10.3*
Total exhaust	22	7.1 ± 0.7	7.8 ± 1.9	48.6 ± 3.8#	115.2 ± 14.1**	99.1 ± 19.8***
Nonpregnant group						
Clean air	6	3.2 ± 0.3	6.0 ± 0.5	42.8 ± 4.3	215.1 ± 20.7	78.4 ± 4.3
Filtered exhaust	6	3.0 ± 0.4	5.5 ± 0.4	44.2 ± 3.0	212.1 ± 20.5	79.3 ± 6.8
Total exhaust	6	2.7 ± 0.2	5.7 ± 0.4	44.1 ± 1.2	203.5 ± 14.7	77.9 ± 4.8

Values are expressed as mean ± SD. *p*-Values were corrected by Bonferroni's inequality.

*Different from clean air, *p* < 0.05; ***different from clean air, *p* < 0.01; **different from clean air, *p* < 0.001; #different from filtered exhaust, *p* < 0.05.

compared to that of fetal ovaries from the control group.

There were six pregnant rats in the testosterone-administered group 1 at the beginning of the experiment. At the end of the experiment, three of these pregnant rats had only implantation traces in their uteri. We analyzed data excluding these rats. Litter sizes ranged from 7 to 10, and the average litter sizes in testosterone groups 1 and 2 were 8.33 and 9.00, respectively. The number of fetuses and the sex ratio of litters in testosterone groups 1 and 2 were 25 (male:female = 13:12, males = 52.0%) and 27 (17:10, 63.0%), respectively. The implantation rates in testosterone groups 1 and 2 were 83.3 and 96.4, respectively.

The fetal parameters in the testosterone-administered groups, such as anogenital distance and the average weight of the thymus, showed the same tendencies as those in the diesel-exhaust-exposed pregnant rat groups and the aromatase inhibitor group (Table 1). The average weight of the placenta from the testosterone groups tended to be lower than that from the other pregnant rat groups. The average body weight in testosterone group 1 tended to be lower than that in the other pregnant rat groups. Differentiation of the fetal testes was delayed or markedly disturbed compared with the testes from the exhaust-exposed groups and the control group. Differentiated Sertoli cells were seldom detected. Primordial germ cells and undifferentiated cells were seen in the seminiferous tubules. The differentiation stage of fetal ovaries from the testosterone groups was obviously delayed compared to that of fetal ovaries from the exhaust-exposed groups and the control group. Numerous germ cells were growing in the primary medulla in the fetal ovary (Figure 12).

The maternal testosterone levels in aromatase inhibitor and testosterone groups were higher than those in the control pregnant rat group (Table 2). Estradiol and progesterone levels in the aromatase inhibitor and testosterone groups were lower than those in the control pregnant rat group (Table 2). The urinary excretion of 17-KS increased markedly in the groups administered these agents (7.9 ± 0.4 , 8.5 ± 0.6 , and 8.4 ± 0.7 $\mu\text{g/ml}/\text{CRE}$ for the aromatase inhibitor group, testosterone group 1, and testosterone group 2, respectively) compared to the excretion of the exhaust-exposed groups and control group. Urinary excretion of 17-OHCS in the aromatase inhibitor group, testosterone group 1, and testosterone group 2 was 11.9 ± 1.2 , 11.9 ± 0.6 , and 11.7 ± 0.7 $\mu\text{g/ml}/\text{CRE}$, respectively.

Discussion

Our study provides evidence for the first time that inhalation of diesel exhaust during

pregnancy masculinizes fetuses through accumulation of testosterone in mother rats. These experiments also showed that the elevated testosterone levels did not result from accelerated maternal adrenal functions. The fetoplacental-ovarian unit and the inhibition of aromatase activities and synthesis caused by inhalation might have played essential roles in the accumulation of testosterone.

The tissue separating the anus and genital papilla differentiates into the scrotum in genetic males under the influence of testosterone. The length of this tissue (anogenital distance) is a sensitive bioassay for exposure to androgen during prenatal life (22–24). The same effect of testosterone on anogenital distance as previously reported (22–24) was also obtained in the testosterone-administered groups in the present study. This study also showed that the anogenital distance was significantly longer in both males and females in the exhaust-exposed groups and that maternal testosterone levels were higher in the exhaust-exposed groups than in the control group. The testosterone levels of both male and female fetuses are correlated with the levels of maternal testosterone (25). Although we did not measure serum testosterone levels of fetuses in these experiments, it seems reasonable to speculate that the elevated serum levels of testosterone in exhaust-exposed rats were

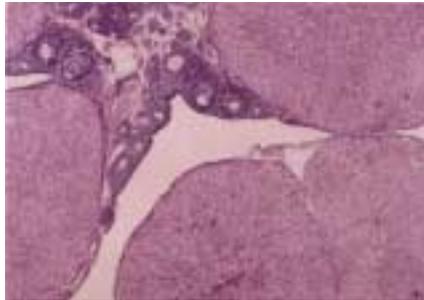


Figure 9. Ovary from control rat on day 20 of pregnancy. The large pregnant *corpora lutea* occupied most of the ovary, and around these *corpora lutea* there were many primary and secondary follicles and the interstitial glands. Magnification x40.

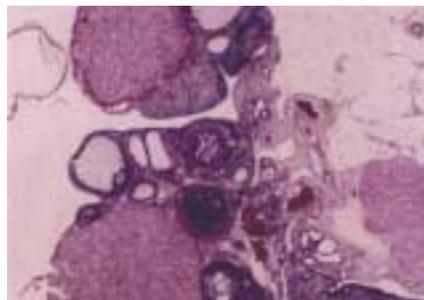


Figure 10. Ovary in the diesel-exhaust-exposed rats on day 20 of pregnancy. The pregnant *corpora lutea* were smaller than those of the control rats. Some of these pregnant *corpora lutea* showed regressive changes. Magnification x40.

responsible for the increased anogenital distance in fetuses from the exhaust-exposed groups.

Excessive maternal testosterone detected in this experiment was not the result of elevated adrenal function. During pregnancy, some serum hormone levels, such as the levels of testosterone, estradiol, progesterone, ACTH, and LH, are different from those in mature nonpregnant females to maintain pregnancy, grow fetuses, and prepare for delivery and milk production. The levels of testosterone, estradiol, progesterone, and ACTH were markedly elevated in all three pregnant groups (total-exhaust-exposed, filtered-exhaust-exposed, and control) in the present study, too. Serum testosterone levels of mother rats exposed to diesel exhaust were significantly higher than those of the control group. Sex steroid hormones are secreted by the placenta, the gonads, and adrenal glands (both maternal and fetal) during pregnancy. In these experiments, the weight of the placenta did not increase in the exposed groups.

As for sex steroid hormones from the gonads, the ovaries were rather small in the exposed groups. The secretion of testosterone

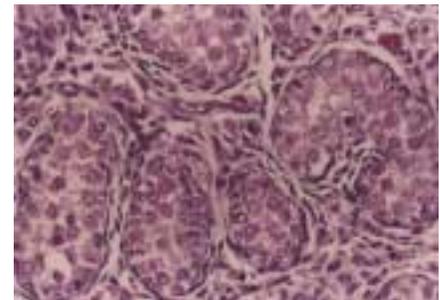


Figure 11. Fetal testis from aromatase inhibitor-administered group on fetal day 20. There were few differentiated Sertoli cells. Swollen primordial germ cells and undifferentiated cells were seen in the seminiferous tubules. Interstitial cells in the unspecialized connective tissue between the seminiferous tubules were not developed. Magnification x200.

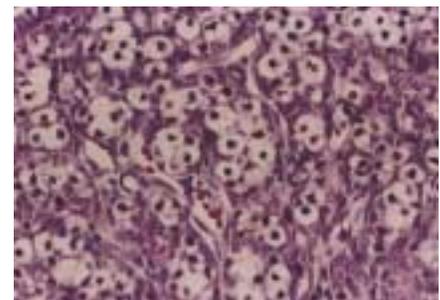


Figure 12. Fetal ovary from testosterone-administered group on fetal day 20. There were numerous germ cells growing in the primary medulla. The differentiation stage of the fetal ovaries was delayed compared to that of fetal ovaries from the control group and diesel-exhaust-exposed groups. Magnification x200.

from the fetal testis was not the major source of elevated sex hormones because the number of interstitial cells of the fetal testis from exhaust-exposed rats, which control testosterone secretion, was not large. Furthermore, adrenal gland function, as indicated by urinary excretion of 17-OHCS, which has been used clinically as a marker of cortisol secretion from the adrenal cortex, did not differ among the pregnant rat groups. Adrenal gland function was thus enhanced because of pregnancy, not because of the various treatments. Urinary excretion of 17-KS—a metabolite of androgens and a marker of adrenal cortex and gonad functions—increased in the groups treated with aromatase inhibitor or testosterone. Although the differences among exposed groups were not significant, 17-KS increased slightly in the group exposed to total exhaust. If the accumulation of testosterone had been marked in exhaust-exposed pregnant rats, urinary excretion of 17-KS would have reflected the excessive levels of testosterone.

The normally functioning fetal adrenal cortex supplies large quantities of dehydroepiandrosterone sulfate (DHEAS), the greater part of which is 16 α -hydroxylated by the fetal liver. DHEAS and 16 α -hydroxydehydroepiandrosterone are desulfated and metabolized further by 3 β -hydroxysteroid dehydrogenase, 17 β -hydroxysteroid dehydrogenase, or both to produce potent androgens in the placentas of humans. However, if the subsequent conversion to estrogens does not occur, free androgens, such as androstenedione, 16 α -hydroxyandrostenedione, testosterone, and 16 α -hydroxytestosterone, accumulate to levels equivalent to the daily excretions of estrogens by normal pregnant women, as seen in cases of aromatase deficiency. Aromatase deficiency causes excess circulation of testosterone and results in virilization (18–21). We found lengthening of the anogenital distance, one of the characteristics of virilization, in the aromatase inhibitor-administered group as well. Although fetal adrenal cortex functions are not so active in animals that have a short gestation period, such as rats and rabbits (38), the fetal hypothalamo-pituitary-adrenal system responds to the abnormal maternal conditions during the late gestational period in rats (39,40).

Nitric oxide inhibits aromatase both by decreasing mRNA for the enzyme and by an acute, direct inhibition of enzyme activity (20,21). Inhalation of diesel exhaust causes uptake of the nitric oxide included in the emission itself and also generates nitric oxide endogenously through the chain reactions of oxidation. Another possible cause of aromatase deficiency in exhaust-exposed mothers during pregnancy is the reduction of

ovarian functions. In rats, progesterone (38) and aromatase activities (41–43) have been detected at remarkably high levels in pregnant luteal cells in ovaries during pregnancy. In pregnant rats exposed to diesel exhaust, serum progesterone levels were low, and the size and the number of the pregnant corpora lutea were small. The serum levels of LH, which is needed to maintain the pregnancy, tended to be reduced. Nitric oxide inhibits gonadotropin-releasing-hormone-stimulated luteinizing hormone release (44,45). Although they were not measured in these experiments, aromatase activities might have been inhibited by nitric oxide directly or indirectly through the reduction of ovarian function. The role of aromatase activities of the fetus in the conversion of maternal testosterone to estrogen is likely to be minor. Aromatase activities are detectable at low levels in both the testis and ovary of fetal mammals (46–48).

Regarding the influence of the intrauterine position on the anogenital distance, as shown in Figure 7, the anogenital distance was influenced by exhaust inhalation, but intrauterine position did not seem to affect the anogenital distance. In the control group, the anogenital distance tended to be longer in 2M females than in 0M and 1M females, although the influence of intrauterine position on the anogenital distance was not significant. In contrast, 0F males tended to show longer anogenital distances than 1F and 2F males in the exposed groups. There was no such difference in the control group (Figure 8). Testosterone levels in female fetuses with males located caudally in the uterus were higher than those in females not so positioned because of the transport of steroids between fetuses (23,25,49,50). Testosterone levels from male fetuses were also affected by the presence of females (51–53). vom Saal et al. (23) suggested that small differences in the concentration of steroids during fetal life lead to marked differences in secondary sexual characteristics in both males and females (49–51). It might be necessary to consider this point carefully when assessing other parameters, such as postnatal reproductive traits and sexual performance.

It is reasonable to assume that deficiency of aromatase caused by nitric oxide resulted in the accumulation of testosterone, masculinization of fetuses, and interference with the differentiation of the testis, ovary, and thymus. The fetoplacental-ovarian unit played an essential role in the excessive production of androgens.

Microscopic examination revealed that differentiation of the fetal testis was delayed or disturbed. There were fewer Sertoli cells in the testes of fetuses from exhaust-exposed pregnant rats. The Sertoli cell population in

the adult animal is determined during the perinatal period; following their initial appearance in the early stages of organogenesis, the differentiating Sertoli cells undergo rapid proliferation with a peak of cell division on day 20 of gestation. Thereafter, proliferation slows down and ceases in pups on day 15 after birth (54–56). It is also known that FSH stimulates Sertoli cell proliferation (56–58). Fetuses have high circulating concentrations of FSH during the late fetal stage (59,60). A reduction of fetal FSH secretion could have occurred and interfered with the differentiation of undifferentiated cells. There have been few reports of adverse effects of excessive testosterone on developing male reproductive organs. In adults, testicular atrophy occurs due to the inhibition of the hypothalamic-pituitary axis affected by pharmacologic doses of androgenic agents (61). Excessive testosterone may disrupt differentiation of Sertoli cells by inhibiting the development of the hypothalamic-pituitary-testis axis in fetuses.

As for the differentiation of the ovary, the differences were less marked among the various groups. The influence of excessive steroid hormones in the ovaries may be different from that in the testes because the development of functional hormonal receptors is slower in fetal ovaries (62–64). Exposure of females to androgens or estrogens during fetal or neonatal life disrupts the mechanisms that control cyclic secretion of the various hormones. During the critical period of development that extends from day 18 of pregnancy until 8–10 days of life, the hypothalamic structures believed to be involved in the control of normal cyclic hormone secretion are undergoing neuronal maturation. Disruption or modification of hypothalamic maturation has a permanent effect that results in a malelike or acyclic pattern of hormone release and infertility (65–69). Further investigations are needed to show whether the slight delay in the differentiation of the ovary during fetal life observed in our experiments leads to marked differences in reproductive abilities, such as delay of puberty, lengthening of the estrous cycle, and accelerating the decline in reproductive performance.

Thymus weight was significantly lower in exposed groups, and histologic examination of the thymus showed that there were fewer cells in the thymuses of these fetuses. On fetal days 17–18, a certain population of cells exhibits a high proliferative rate, and the first mature thymocytes appear on fetal day 20 (70–72). The fetal thymus on day 20 of gestation looked stable in this study. Testosterone-binding cells have been found in the thymus of 18-day-old fetuses, and testosterone can influence the function of specific thymic

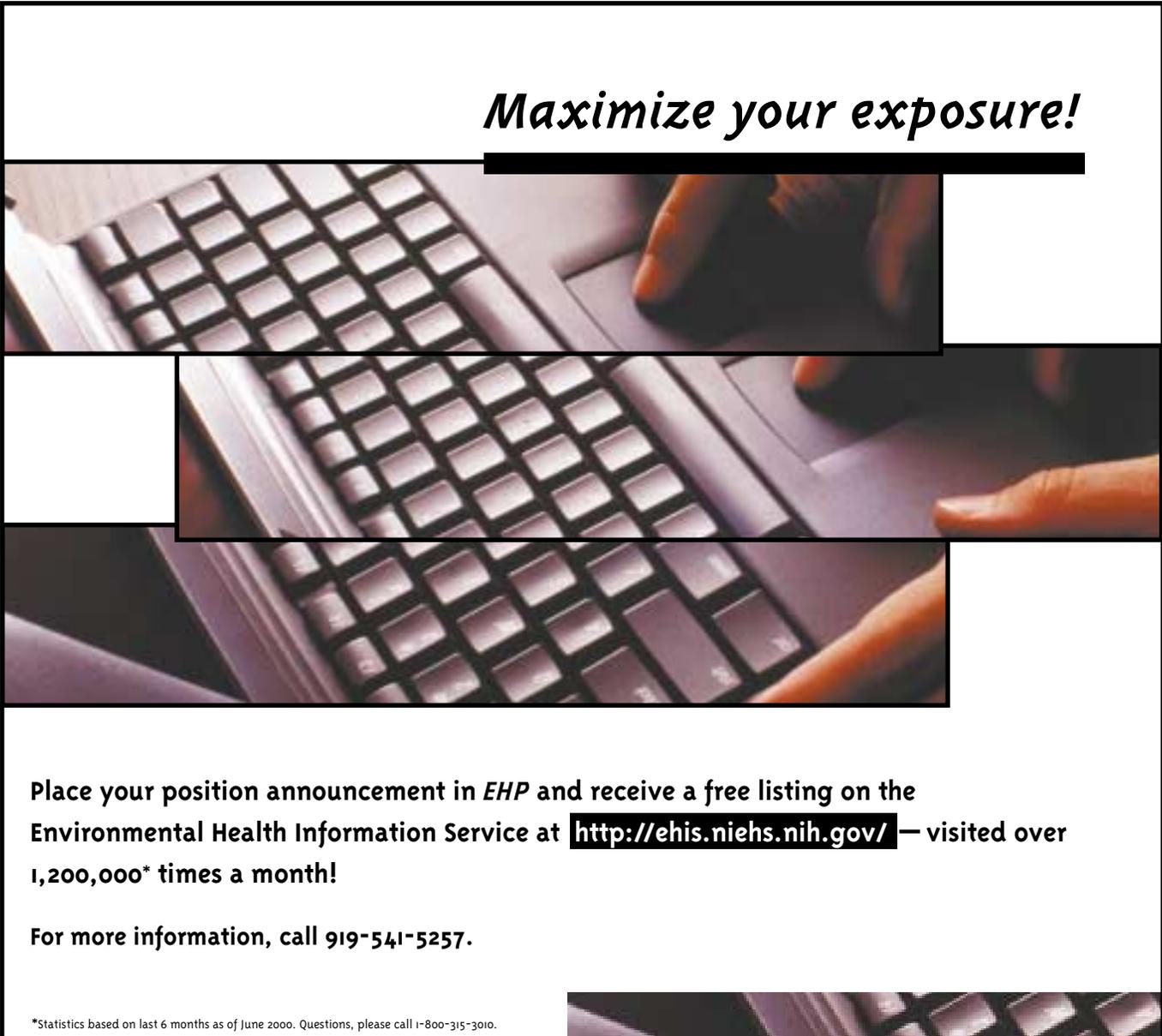
epithelial cells not only directly by acting on the thymus cells but also indirectly by modulating the function of the thymus epithelial cells that bind testosterone (73). The high levels of testosterone might have influenced cellular differentiation and proliferation in the fetal thymus. There is a strong correlation between immunodeficiency of the thymus, especially of suppressor T cells, and elevated serum IgE (28–29). As recently reported, diesel exhaust particles, carbon black (74,75), and airborne house dust (76) have adjuvant effects on IgE production. There could be some connection between the immunosuppression that occurs during the fetal period because mothers are exposed to polluted air and the increased prevalence of atopic diseases in infants. Further studies are needed to show whether delayed or suppressed differentiation of the thymus in fetal life has a connection with immunodysfunction postnatally.

Our study addressed the possibility that inhalation of diesel exhaust disrupts differentiation through the accumulation of endogenous sex steroid hormones. The gaseous phase must include toxicants that induce accumulation of steroid metabolites because mothers and fetuses in both exhaust-exposed groups showed almost the same reactions to the exposure. Further investigations are needed to identify the substances responsible for the endocrine disruption and to clarify the possible relationship between the developmental defects caused by prenatal exposure to diesel exhaust and reproductive and immune dysfunctions in later life.

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